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Particulate Methane Monooxygenase Genes in Methanotrophs

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A 45-kDa membrane polypeptide that is associated with activity of the particulate methane monooxygenase (pMMO) has been purified from three methanotrophic bacteria, and the N-terminal amino acid sequence was found to be identical in 17 of 20 positions for all three polypeptides and identical in 14 of 20 positions for the N terminus of AmoB, the 43-kDa subunit of ammonia monooxygenase. DNA from a variety of methanotrophs was screened with two probes, an oligonucleotide designed from the N-terminal sequence of the 45-kDa polypeptide from *Methylococcus capsulatus* Bath and an internal fragment of *amoA*, which encodes the 27-kDa subunit of ammonia monooxygenase. In most cases, two hybridizing fragments were identified with each probe. Three overlapping DNA fragments containing one of the copies of the gene encoding the 45-kDa pMMO polypeptide (*pmoB*) were cloned from *Methylococcus capsulatus* Bath. A 2.1-kb region was sequenced and found to contain both *pmoB* and a second gene, *pmoA*. The predicted amino acid sequences of these genes revealed high identity with those of the gene products of *amoB* and *amoA*, respectively. Further hybridization experiments with DNA from *Methylococcus capsulatus* Bath and *Methylobacter albus* BG8 confirmed the presence of two copies of *pmoB* in both strains. These results suggest that the 45- and 27-kDa pMMO-associated polypeptides of methanotrophs are subunits of the pMMO and are present in duplicate gene copies in methanotrophs.

Methanotrophs are a group of gram-negative bacteria that utilize methane as their sole source of carbon and energy. The initial transformation involves the conversion of methane into methanol by methane monooxygenase (MMO): $CH_4 + O_2 + NADH + H^+ \rightarrow CH_3OH + H_2O + NAD^+$. Methanol is then further oxidized to formaldehyde, which is either assimilated into biomass or oxidized to carbon dioxide. The MMO exists in two forms, a cytoplasmic or soluble form (sMMO) and a membrane-bound or particulate form (pMMO). The sMMO is found in only a subset of the methanotrophs, while the pMMO is present in all known methanotrophs (18, 21). The sMMO has been purified from several methanotrophs and has been characterized biochemically and genetically (8, 15, 16, 29, 30, 36). The pMMO is less well studied than the sMMO and has never been reproducibly purified to homogeneity (31, 39), possibly because of the instability of the pMMO upon removal of the polypeptides from the membrane lipids. Therefore, much is still unknown about the structure of the pMMO. It is important to obtain a more fundamental understanding of the pMMO because of the importance of methanotrophs in the global methane cycle (32) and the fact that the majority of known methanotrophs contain only the pMMO. Furthermore, methanotrophs have been proposed for the in situ bioremediation of sites contaminated with small halogenated solvents (28, 41), and the pMMO can degrade halogenated hydrocarbons, including trichloroethylene (12).

Some information concerning the pMMO has come from differential expression studies of methanotrophs that contain both pMMO and sMMO. It is known that for cells able to express both forms of the MMO, the ratio of copper to biomass is significant in controlling the relative expression of the sMMO and pMMO (35, 40). Under low copper/biomass ratios, the sMMO is the predominant MMO expressed, while at high copper/biomass ratios, MMO activity is associated with the membrane fractions. When the soluble and particulate fractions of cells grown under increasing copper/biomass ratios are analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the known sMMO polypeptides decrease in intensity in the soluble fraction while two polypeptides of approximately 45 and 27 kDa appear in the particulate fractions concomitant with the appearance of pMMO activity (9, 10, 12). Polypeptides of similar sizes were found as major species in a partially purified pMMO preparation from *Methylococcus capsulatus* Bath (39), providing further evidence that these polypeptides may be pMMO subunits. Additional information has come from studies using acetylene, a suicide substrate for both the sMMO and the pMMO (2, 17, 19, 34). In cells containing the sMMO, [^{14}C]acetylene specifically labels the alpha subunit of the hydroxylase (34). In cells containing the pMMO, [^{14}C]acetylene specifically labels a membrane polypeptide of approximately 27 kDa, which is assumed to be the 27-kDa polypeptide mentioned above (12). pMMO activity has been obtained in purified membrane preparations (31) and in a partially purified preparation extracted from membranes (39) by an assay based on the ability of MMO to oxidize propene. For these assays, NADH is commonly used as a source of reducing equivalents, but the electron donor in vivo is not known (31, 33).

Recent studies of another enzyme, the ammonia monooxygenase (AMO), have also provided information relevant to the pMMO. The AMO shares many characteristics with the pMMO. Both enzymes have similar substrate ranges, including their primary substrates, methane and ammonia, although the apparent K_m values for these substrates differ (2, 42). Both enzymes are inhibited by acetylene, and in both enzymes, [^{14}C]acetylene labels a membrane polypeptide of approximately 27 kDa (20). In addition, a 43-kDa polypeptide copurifies with the 27-kDa acetylene-binding polypeptide in *Nitrosomonas europaea* (26), and these are thought to be analogous

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to the 27- and 45-kDa polypeptides associated with the pMMO. Substantial evidence suggests the 27- and 43-kDa polypeptides are subunits of the AMO (20, 26). Both enzymes are highly unstable after cell lysis, but activity in vitro is stabilized by copper for both the AMO (14) and the pMMO (31), and copper has been suggested to be part of the active site of both enzymes (14, 31). It has been reported recently that the pMMO may contain unusual trinuclear copper clusters that are responsible for the activation of oxygen (31). Although nothing was known about the genetics of the pMMO prior to the present study, the gene encoding the acetylene-binding subunit of the AMO (*amoA*) has been cloned from *N. europaea* and sequenced (26). The sequence predicts the actual size of AmoA to be 32 kDa. The second gene encoding the 43-kDa AMO subunit was discovered downstream and labeled *amoB*, and it has also been sequenced (3, 26). In these same studies, it was shown that *N. europaea* contains two gene copies of *amoA* and *amoB*. In this paper, we report the presence in methanotrophs of two copies of genes similar to *amoA* and *amoB* of *N. europaea*. Furthermore, we have cloned and sequenced one copy of the genes for the 45- and 27-kDa putative pMMO subunits.

MATERIALS AND METHODS

Growth of methanotrophs. Methanotrophs were grown on nitrate mineral salts medium in batch culture with copper added as $CuSO_4 \cdot 5H_2O$ (43). For *Methylobacter marinus* A45 (previously called *Methylomonas* sp. strain A45) (5, 22), a sterile solution of NaCl was added for a final concentration of 1% (wt/vol). The cells were shaken at 200 rpm under a methane-air headspace (approximately 1:3 [vol/vol]) at 1 atm (101.29 kPa) of pressure. *Methylobacter albus* BG8 (previously called *Methylomonas albus* BG8), *Methylocystis parvus* OBBP, and *Methylosinus trichosporium* OB3b (43) were grown at 30°C, while *Methylococcus capsulatus* Bath (43) was grown at 45°C and *Methylobacter marinus* A45 was grown at 37°C. The cells were grown to the late exponential phase and harvested.

DNA purification and hybridization. Chromosomal DNA was isolated from the methanotrophs by a modified version of the Marmur technique (25). Cells were first pelleted by centrifugation at 5,000 rpm. The cells were then washed once with saline-EDTA (0.15 M NaCl, 0.1 M EDTA [pH 8.0]) and resuspended in 10 ml of saline-EDTA to which 10 mg of lysozyme per ml was added. The suspensions were frozen at −70°C. Before thawing, 10 ml of Tris-SDS (0.1 M Tris, 1% [wt/vol] SDS, 0.1 M NaCl [pH 9.0]) with 1 mg of proteinase K per ml was added, and the solution was placed in a 50°C water bath for 30 min or until the solution became translucent. The DNA was extracted with a phenol-saline-EDTA solution and then precipitated with isopropanol and resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]).

Plasmid DNA was isolated from *Methylobacter albus* BG8 as follows. Cells were washed once and then resuspended in TE buffer. The cells were then lysed with 4% SDS (wt/vol) in TE buffer (pH 12.4). After 30 min of incubation at room temperature, a neutral buffer (2 M Tris [pH 7.0]) and a salt solution (5 M NaCl) were added. The resulting suspension was then placed on ice overnight. The samples were then centrifuged at 39,000 × *g* for 20 min at 4°C. The supernatant was removed, and 0.6 volume of isopropanol was added. The solution was centrifuged at 12,000 × *g* for 20 min at 23°C, and the pelleted plasmid DNA was resuspended in TE buffer and analyzed on 0.7% agarose gels in a Tris-acetate buffering system.

Both chromosomal and plasmid DNA were digested with restriction enzymes as recommended by the supplier (New England Biolabs, Inc., Beverly, Mass.). The digested DNA was separated by standard procedures on 0.7% agarose–Tris-acetate gels, and these were prepared for hybridization as described by Meinkoth and Wahl (27).

The oligonucleotide probe AC10 [5′-ATG-CG(A,G,C, or T)-AC(A,G,C, or T)-AT(A,C, or T)-CA(C or T)-TGG-TA(C or T)-GA-3′] was based on the amino acid sequence MRTIHWY of the N-terminal region of the 45-kDa polypeptide from two methanotrophs (see Table 1) and had 192-fold redundancy. It was end labeled with T4 polynucleotide kinase and 5′-[γ-^{32}P]ATP as described by Sambrook et al. (37). The probe was then hybridized to dried gels overnight at 42°C with a standard hybridization solution (0.5% [wt/vol] SDS, 1 M NaCl, 0.1 M sodium citrate, 0.5% [wt/vol] powdered milk). Excess probe was removed with a wash solution of 0.1% SDS, 75 mM NaCl, and 7.5 mM sodium citrate at 42°C. The dried gels were then exposed to X-ray film.

Hybridizations were also performed with the cloned *Methylococcus capsulatus* Bath 0.9-kb *Pst*I fragment and a fragment of the *N. europaea amoA* gene. For the latter, the PCR was used to amplify a 693-bp internal fragment of the gene. Primers used were AMO1 (GCACTTTATGCTGCTGGC) and AMO2 (GATC CCCTCTGGAAAGCC), which correspond to regions of *amoA* at positions 380 to 398 and 1073 to 1056, respectively, of the published sequence (26). Amplification was carried out on a Hybaid thermal cycler (Combi TR-2) in 50-μl volumes. Each tube contained 0.1% (wt/vol) Triton X-100, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 100 μM each deoxynucleoside triphosphate, 50 pmol of each primer, 1 U of *Taq* polymerase (Promega, Madison, Wis.), and approximately 1 ng of template DNA. Reaction conditions were the following (for a total of 30 cycles): denaturation, 94°C for 1 min; annealing, 50°C for 1 min; polymerization, 72°C for 1 min. The *amoA* gene was cloned from DNA of *N. europaea* (NCIMB11850), kindly supplied by J. Prosser, University of Aberdeen, with the T/A cloning kit (Invitrogen, San Diego, Calif.) as described in the vendor's instructions. Plasmids containing inserts were purified by the method of Saunders and Burke (38), and the insert size was checked after digestion with *Eco*RI. The identity of the 693-bp *amoA* gene fragment was confirmed by sequencing. Hybridizations with the *Pst*I fragment and with the *amoA* probe were carried out with DNA blotted to nylon membranes (Hybond N; Amersham) as outlined by Sambrook et al. (37), with hybridizations performed in 6× SSC–0.5% SDS at 65°C and subsequent washes in 2× SSC–0.1% SDS at 55°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Both probes were labeled by the random primer method with a kit from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and isolated fragments prepared as described by Sambrook et al. (37).

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MC      HGEKSQAAFMRMRTIHWYDL
        |||  |||||||||||||•||
A45     HGEXSQAAFMRMRTIHWFDL
        |||  |||||||||||||•||
BG8     HGEXSQAAFMRMRTIHWYDL
        |||  ||   |  ||||•  ||||
AmoB    HGERSQEPFLRMRTVQWYDL
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FIG. 1. N-terminal amino acid sequence for the 45-kDa polypeptide from methanotrophs and for AmoB of *N. europaea* (26). Vertical bars indicate identical amino acids, dots denote conserved substitutions, and X's indicate unknown amino acids. Abbreviations: MC, *Methylococcus capsulatus* Bath; A45, *Methylobacter marinus* A45; BG8, *Methylobacter albus* BG8.

Protein purification and sequencing. Membrane fractions of *Methylococcus capsulatus* Bath, *Methylobacter albus* BG8, and *Methylobacter marinus* A45 were collected as follows. Cells were centrifuged at 7,500 × *g* for 10 min and then washed with 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (pH 7.0). The cells were passed three times through a French pressure cell at 137 MPa. Any remaining whole-cell debris was removed by centrifugation at 10,000 × *g* for 15 min. The supernatant was poured off and recentrifuged at 150,000 × *g* for 90 min. The resulting membrane pellets were resuspended in a sample buffer solution (50% [wt/vol] urea, 0.2% [wt/vol] bromophenol blue, 4% [wt/vol] SDS, 100 mM Tris [pH 6.8]).

SDS-PAGE was performed with the Xcell II gel system (Novex, Inc., San Diego, Calif.). Prepackaged 1.0-mm-thick Tris-glycine gels with 10 to 27% polyacrylamide gradients were used for protein separation. The gels were prerun with SDS-urea gel loading buffer to remove any free radicals that may modify the N terminus of desired polypeptides. The running buffer contained 25 mM Tris, 250 mM glycine, 0.1% (wt/vol) SDS, and 0.1 mM thioglycolate. The system was

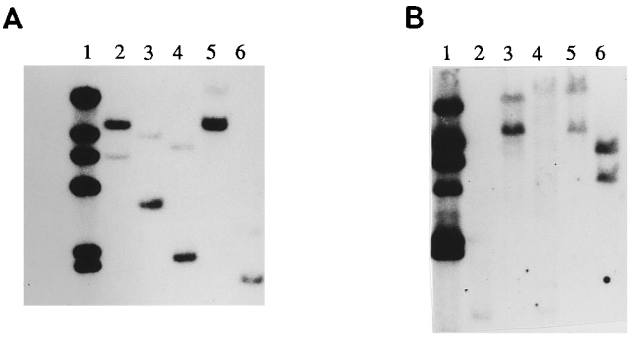

FIG. 2. Hybridization of AC10 to restriction enzyme digests of *Methylobacter albus* BG8 (A) and *Methylococcus capsulatus* Bath (B) chromosomal DNA. (A) Lanes: 1, molecular weight standards (lambda DNA digested with *Hin*dIII; sizes, 23, 9.4, 6.6, 4.4, 2.3, and 2.0 kb); 2, *Pst*I digest; 3, *Hin*dIII digest; 4, *Eco*RI digest; 5, *Bgl*II digest; 6, *Bst*YI digest. (B) Lanes: 1, molecular weight standards (same as those for panel A); 2, *Pst*I digest; 3, *Bam*HI digest; 4, *Sal*I digest; 5, *Hin*dIII digest; 6, *Eco*RI digest. Lanes 2 to 6 contain approximately 10 μg of DNA.

TABLE 1. Sizes of DNA fragments of *Methylococcus capsulatus* Bath and *Methylobacter albus* BG8 that hybridized to the probes, AC10 (5′ end of *pmoB*), the 0.9-kb *pmoB Pst*I fragment, and *amoA*

Strain	Enzyme	Size(s) (kb) of fragments that hybridized to[a]:		
		AC10	0.9-kb *Pst*I (*amoB*)	*amoA*
M. capsulatus Bath	*Bam*HI	**13, 9.3**	**13, 9.3**	
	*Bgl*II		12	
	*Eco*RI	**7.2, 4.1**	**7.2, 4.1,** (2.2, 1.0)	
	*Bst*YI		**6.9, 4.0**	
	*Hin*dIII	**12, 9**	**12, 9**	
	*Pst*I	**0.9**	**0.9**	
	*Sal*I	**1.2, 0.9**	**0.9,** 0.4, (0.5)	
M. albus BG8	*Bam*HI		4.3, (**5.4**)	**5.4**
	*Bgl*II	14, (23)	7.0, 2.3, (**5.4,** 4.5)	**5.4**
	*Eco*RI	**2.1,** (8.2)	**2.1,** 0.6, (3.8, 1.8, **1.1**)	**1.1**
	*Bst*YI	**1.8, (2.3)**	**1.8, (2.3, 1.6,** 0.95, 0.71)	**1.6**
	*Hin*dIII	**4.0,** (9.5)	**4.0, (5.5,** 3.5)	**5.5**
	*Pst*I	14, (7.3)	0.9, (**3.3,** 3.0)	**3.3**
	*Sal*I		3.3, 0.4, (**7.9,** 5.8)	**7.9**

[a] Fragments of the same size for more than one probe are in boldface type. Fragments showing light hybridization are in parentheses.

operated at 125 V for 90 min. After electrophoresis, the polypeptides were transferred to Immobilon P membranes (Millipore Corp., Bedford, Mass.) by use of a Bio-Rad Trans-Blot cell. The polypeptides were transferred to the membranes overnight at 100 V in a transfer buffer containing 12 mM Tris, 96 mM glycine, 10% (vol/vol) methanol, and 0.5 mM dithiothreitol. The membranes were soaked in water overnight to remove residual glycine. Bands of approximately 45 kDa were excised. N-termini were sequenced by Edman degradation on ABI 476 and 477 pulsed liquid protein sequencers (Applied Biosystems, Inc., Foster City, Calif.) by the Biopolymer Synthesis & Analysis Resource Center at the California Institute of Technology. From the sequence found for *Methylococcus capsulatus* Bath, the oligonucleotide probe AC10 was synthesized at the Microchemical Facility at the California Institute of Technology.

Cloning of *pmoA* and *pmoB* from *Methylococcus capsulatus* Bath. Fractions of the restriction endonuclease-digested *Methylococcus capsulatus* Bath and *Methylobacter albus* BG8 chromosome that hybridized with the AC10 oligonucleotide were isolated from agarose gels as described by Sambrook et al. (37). Each fraction was ligated with the vector pRK310 (13) to generate partial clone libraries. These were used to transform *Escherichia coli*, and approximately 1,000 colonies of each were screened by hybridization to the AC10 probe. Only one of these libraries, made with *Methylococcus capsulatus* Bath chromosomal DNA digested with *Pst*I (0.8- to 1.5-kb fraction), resulted in colonies that hybridized to the probe. Plasmids were isolated from four separate colonies, and all contained the same 0.9-kb insert. The 0.9-kb *Pst*I fragment was recloned in pAYC63 (7) and sequenced on both strands at the Sequencing Core Facility at the University of California at Los Angeles.

Other *Methylococcus capsulatus* Bath DNA fragments were also isolated as described above, except that the 0.9-kb *Pst*I fragment was used as a probe. The partial libraries screened were made with chromosomal DNA digested either with *Eco*RI (0.8- to 1.5-kb or 2.0- to 3.0-kb fractions) or with *Kpn*I (2- to 3-kb or 3.5- to 5-kb fractions). Portions of the cloned fragments were sequenced by cycle sequencing with the pUC universal and reverse primers and primers based on the determined sequences as described in the recommendations of the manufacturer (Taq Dideoxy Terminator Cycle Sequencing Kit; Applied Biosystems). Sequencing reactions were analyzed on an ABI 373A automated sequencer (Applied Biosystems) at the DNA Sequencing Core Facility, California Institute of Technology.

Homology searches. The Wisconsin Genetics Computer Group program (11) and PC/GENE (Genofit SA, Geneva, Switzerland) were used to search for genes and polypeptide sequences with similarity to the cloned gene for the 45-kDa and 27-kDa polypeptides. The Wisconsin package was also used for calculating the hydrophilicity of the primary sequence of the 45-kDa and 27-kDa polypeptides with the Kyte-Doolittle algorithm over a span of 7 residues. The nucleotide sequence corresponding to the *pmoA* and *pmoB* of *Methylococcus capsulatus* Bath was deposited in GenBank under the accession number L40804.

RESULTS

Purification and sequencing of the 45-kDa polypeptide. Two major polypeptides of 45 and 27 kDa are consistently associated with the pMMO (1, 10, 12, 31), and we have observed that the major band on SDS-polyacrylamide gels around 45 kDa contains two polypeptides of approximately 45 and 46 kDa. The membrane polypeptides from three methanotrophs, *Methylococcus capsulatus* Bath, *Methylobacter albus* BG8, and *Methylobacter marinus* A45, were transferred to Immobilon P membranes, and the areas containing the 45- and 46-kDa polypeptides were sequenced. No sequence was obtained for the 27-kDa polypeptide. However, 20 residues were obtained

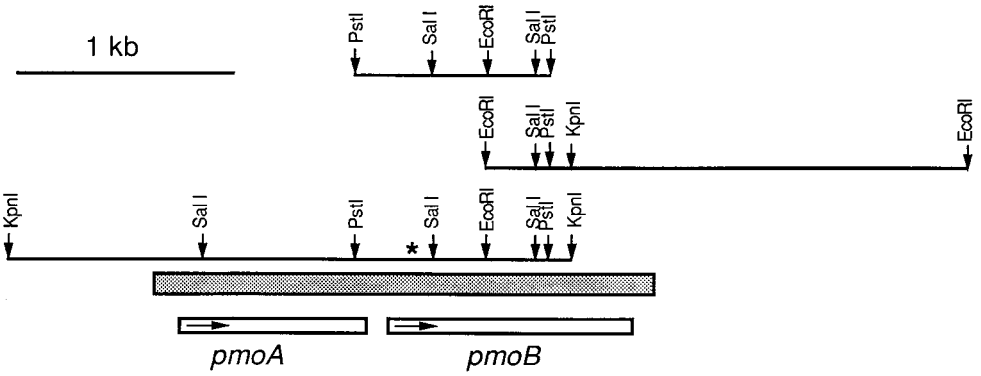

FIG. 3. Physical map of the three overlapping *Methylococcus capsulatus* Bath DNA fragments containing *pmoA* and *pmoB*. The region sequenced is indicated by the stippled box, and the directions of transcription of *pmoA* and *pmoB* are shown by the arrows. The asterisk indicates the sequence corresponding to the AC10 probe.

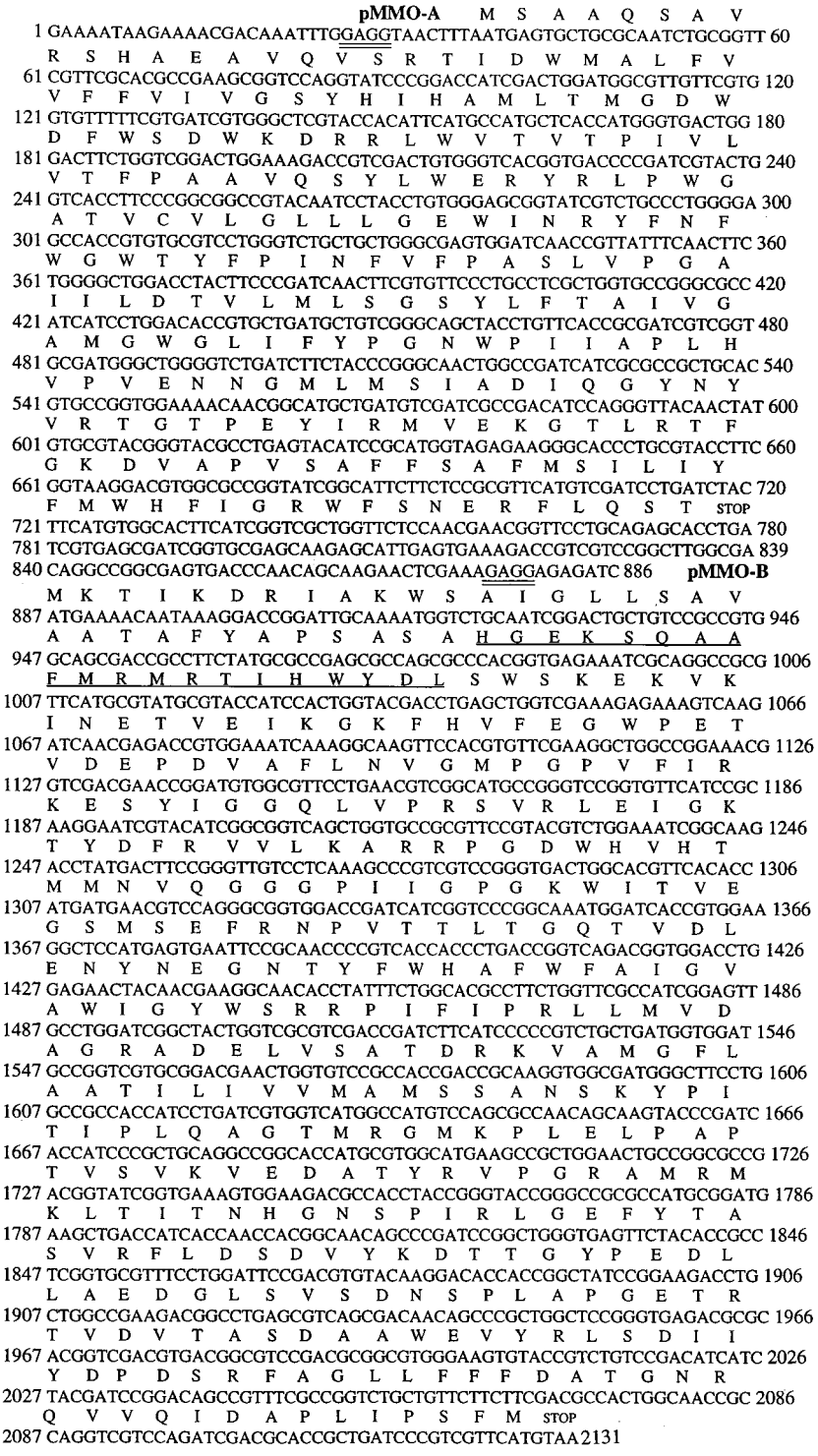

FIG. 4. Sequence of the 2,084-bp region containing *pmoA* and *pmoB*. The translated amino acid sequence is shown above that sequence, the putative Shine-Dalgarno sequence is double-underlined, and the amino acids determined for the 45-kDa polypeptide are underlined.

for the 45- and 46-kDa polypeptides, and they were identical for the strain from which they were isolated. The sequences between strains differed only at the 4th and 18th residues (Fig. 1). For *Methylococcus capsulatus* Bath, the fourth residue was lysine, while in *Methylobacter marinus* A45, the fourth amino acid was unidentified. For *Methylobacter albus* BG8, the fourth residue coeluted with tyrosine but did not have a typical tyrosine spectral shape, suggesting that this residue is an unusual amino acid. In *Methylococcus capsulatus* Bath and *Methylobacter albus* BG8, the 18th residue was tyrosine, while in *Methylobacter marinus* A45, it was phenylalanine.

A search of both protein and DNA databases for polypep-

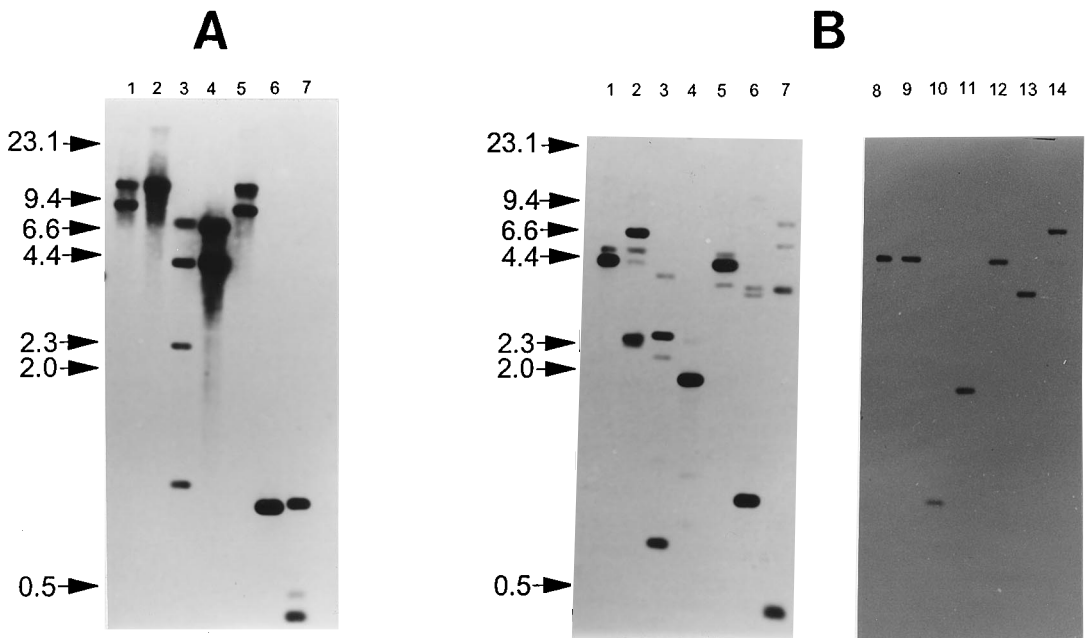

FIG. 5. Hybridization of digests of methanotroph genomic DNA to *pmo* and *amo* probes. (A) *Methylococcus capsulatus* Bath DNA; (B) *Methylobacter albus* BG8 DNA. Lanes: 1 to 7, 0.9-kb *Methylococcus capsulatus* Bath *Pst*I *pmoB* fragment as probe; 8 to 14, 693-bp *amoA* fragment as probe. Restriction digests in lanes: 1 and 8, *Bam*HI; 2 and 9, *Bgl*II; 3 and 10, *Eco*RI; 4 and 11, *Bst*YI; 5 and 12, *Hin*dIII; 6 and 13, *Pst*I; 7 and 14, *Sal*I. All lanes contain approximately 10 μg of DNA.

tides similar to the portion of the 45-kDa polypeptide sequenced in this study identified only one match. As shown in Fig. 1, the N terminus of the gene product of *amoB*, the 43-kDa subunit of AMO, has 14 of 20 residues identical to the N terminus of the 45-kDa polypeptide sequenced from *Methylococcus capsulatus* Bath and *Methylobacter albus* BG8 and 13 of 20 residues identical for the polypeptide from *Methylobacter marinus* A45. We propose to designate the gene encoding this polypeptide *pmoB*, in analogy with *amoB*.

Hybridization of probes to methanotroph DNA. The sequence MRTIHWY of the 45-kDa polypeptide found in *Methylococcus capsulatus* Bath and *Methylobacter albus* BG8 was used to design an oligonucleotide probe, AC10 (see Materials and Methods). Chromosomal DNA from four methanotrophs, *Methylococcus capsulatus* Bath, *Methylobacter albus* BG8, *Methylosinus trichosporium* OB3b, and *Methylocystis parvus* OBBP, representing both major known phylogenetic categories (6), was digested with *Hin*dIII, *Bst*YI, and *Pst*I, separated on gels, and probed with AC10. Since the N-terminal amino acid sequences of PmoB were so similar to those of AmoB, it seemed likely that the 27-kDa pMMO-associated polypeptide found in methanotrophs might also be similar to AmoA, the 27-kDa subunit of AMO; therefore, gels were also probed with an internal fragment of *amoA*. Hybridization to specific DNA fragments was observed for both probes for all four methanotrophs except *Methylocystis parvus* OBBP with AC10, suggesting that genes similar to *amoA* and *pmoB* were present in most of these strains. Hybridization to the *amoA* probe was weaker than that to AC10. However, in most cases in which hybridization was detected, two DNA fragments were identified for at least two restriction digests (data not shown).

The presence of two hybridizing bands in methanotroph DNA suggested that duplicate gene copies might exist for *pmoB* and for a second gene with similarity to *amoA*. This raised the possibility that one set of genes might be plasmid encoded. The only plasmid in a methanotroph that has been characterized is a 65-kb plasmid that has been isolated from *Methylobacter albus* BG8 and for which a restriction map has been determined (24). To determine whether one of the hybridizing fragments was part of this *Methylobacter albus* BG8 plasmid, the plasmid was isolated, digested with *Hin*dIII, *Bst*YI, and *Bam*HI, and separated on gels. Membranes containing the plasmid DNA were hybridized to the AC10 probe, but no hybridization was observed. When the procedure was repeated with intact plasmid DNA that had been denatured with a solution of 150 mM NaCl and 0.5 N NaOH, AC10 did not hybridize to the circular DNA (data not shown).

Cloning and sequencing of *pmoA* and *pmoB* from *Methylococcus capsulatus* Bath DNA. Attempts were made to clone DNA fragments containing *pmoA* and *pmoB* from *Methylococcus capsulatus* Bath and *Methylobacter albus* BG8. Since the hybridization to *amoA* was weak, the initial focus was on cloning fragments containing part of *pmoB*. DNA from these strains was digested with five different restriction enzymes and probed with AC10 (Fig. 2). In most cases, two bands were identified, and their sizes are noted in Table 1. Initially, attempts were made to clone the *Eco*RI fragments from *Methylococcus capsulatus* Bath and the *Bst*YI and *Hin*dIII fragments from *Methylobacter albus* BG8. This was unsuccessful, presumably because of a toxic effect in *E. coli*, as reported for cloning of the *amoA* and *amoB* region (26). We were successful, however, in cloning a 0.9-kb *Pst*I fragment from *Methylococcus capsulatus* Bath. Three separate clones containing this fragment were partially sequenced, and all were identical. One of these was sequenced completely. The sequence revealed an open reading frame of 265 amino acids that extended to one end of the fragment and which contained the N-terminal sequence determined for the 45-kDa polypeptide (Fig. 3 and 4). Therefore, this *Pst*I fragment contained the 5′ two-thirds of *pmoB*. The cloned 0.9-kb *Pst*I fragment was used as a probe against digests of *Methylococcus capsulatus* Bath DNA to identify fragments that might contain the 3′ region of *pmoB*. Seven

restriction digests were screened, and in four of the digests that had been screened previously, at least one of the fragments detected with the *Pst*I probe was the same size as that detected with the AC10 probe (Fig. 5A; Table 1). However, in the *Eco*RI digest, two additional fragments of 2.2 and 1.0 kb hybridized with the *Pst*I probe that did not hybridize with the AC10 probe (Table 1; Fig. 2 and 5A). The 2.2- and 1.0-kb fragments were cloned, and the 5′ regions were partially sequenced. The sequence of the 2.2-kb *Eco*RI fragment matched the overlapping portion of the 0.9-kb *Pst*I fragment exactly, while that of the 1.0-kb *Eco*RI fragment diverged slightly. Therefore, it was assumed that the 2.2-kb *Eco*RI fragment was the one that overlapped the 0.9-kb *Pst*I fragment on the chromosome, and the 5′ region of this fragment was completely sequenced (Fig. 3 and 4). An open reading frame that matched the open reading frame of *pmoB* from the 0.9-kb *Pst*I fragment and terminated within the *Eco*RI fragment was identified. This combined open reading frame contains 414 amino acids and is predicted to encode a polypeptide of 45,540 Da with a 32-residue leader sequence. The N-terminal amino acid of the isolated PmoB is the His at residue 33 of the open reading frame, immediately following the proposed leader sequence (Fig. 1 and 6B). The 300 bp of overlap between the *Pst*I and *Eco*RI fragments showed 100% identity at the nucleotide sequence level. The 1.0-kb *Eco*RI fragment identified presumably contains the 3′ portion of the second copy of *pmoB*, since the preliminary sequence had high similarity (97% identical) to the same region from the 2.2-kb *Eco*RI fragment. A search of both protein and DNA databases revealed only one entry showing significant similarity, that for *amoB* from *N. europaea* (3, 26). The translated amino acid sequence of *pmoB* could be aligned with the translated *amoB* sequence and showed 42.9% identity over the 387-amino-acid overlap that excludes the proposed leader sequences (Fig. 6B). When the PmoB sequence was analyzed on a hydropathy plot with the Kyte-Doolittle algorithm, it was revealed to be hydrophobic and contained two potential membrane-spanning segments in the mature polypeptide in addition to a hydrophobic hypothesized leader sequence (Fig. 7). The pattern observed was similar to that for AmoB, except that a third membrane-spanning region predicted for AmoB at residues 274 to 290 is not conserved in PmoB (3).

The region upstream of *pmoB* was sequenced by cloning an overlapping *Kpn*I fragment as follows. The sequence of *pmoB* revealed an internal *Kpn*I site (Fig. 3 and 4). Probing of blots made with *Methylococcus capsulatus* Bath genomic DNA digested with *Kpn*I with the 0.9-*Pst*I fragment as a probe revealed two fragments of 2.8 and 4.0 kb. Attempts were made to clone both fragments, but only the 2.8-kb fragment was obtained. Restriction mapping showed that this fragment overlapped at the 3′ end with the known restriction map of *pmoB* (Fig. 3). A region that contained the 5′ end of *pmoB* and extended 0.9 kb upstream was sequenced on both strands (Fig. 3 and 4). An open reading frame of 248 amino acids was identified; this open reading frame terminates within the 0.9-kb *Pst*I fragment such that this fragment encodes the final 4 amino acids of the open reading frame. The sequenced region of this clone that overlaps with the *Pst*I fragment (300 bp) showed 100% identity to the previously determined sequence, suggesting that this *Kpn*I fragment overlaps the 0.9-kb *Pst*I fragment in the chromosome. A search of both protein and DNA databases revealed significant identity to only one entry, that of *amoA* (26). The translated amino acids of this open reading frame showed 46.7% identity to those of *amoA* (Fig. 6A), and this gene is proposed to be designated *pmoA*. The hydropathy plot of PmoA is shown in Fig. 7, and it predicts four membrane-spanning regions, with no classical leader sequence at the N terminus. This structure is similar to that predicted for AmoA, except that the third predicted membrane-spanning region is approximately twice as long in PmoA as it is in AmoA (26).

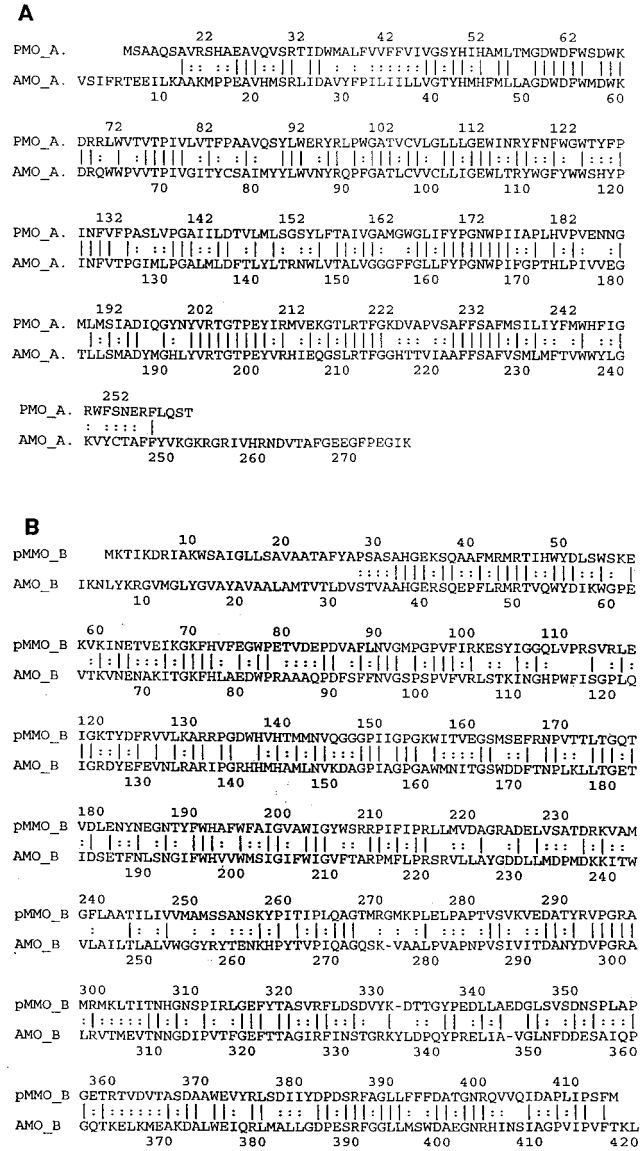

FIG. 6. Comparison of predicted amino acid sequences of *pmo* and *amo* genes. (A) *pmoA* from *Methylococcus capsulatus* Bath (upper sequence) and *amoA* from *N. europaea* (lower sequence [26]); (B) *pmoB* from *Methylococcus capsulatus* Bath (upper sequence) and *amoB* from *N. europaea* (lower sequence). Identical amino acids are marked with vertical lines, and conserved substitutions are marked with dots.

Comparison of sequence and hybridization data for *Methylococcus capsulatus* Bath. The restriction map of the combined 4.4-kb cloned *pmo* region agrees with the sizes of the fragments identified by hybridization with the two *pmoB* probes. No *Bam*HI, *Bgl*II, or *Bst*YI sites were present in this *pmoA* and *pmoB* region, and as expected for each of these digests, at least one fragment larger than the cloned region was identified by hybridization. Both copies of *pmoB* appear to contain an *Eco*RI site in the same region of the genes, since the two cloned *Eco*RI fragments showed similar sequences at the 5′ ends. In addition, only one *Pst*I fragment was identified by

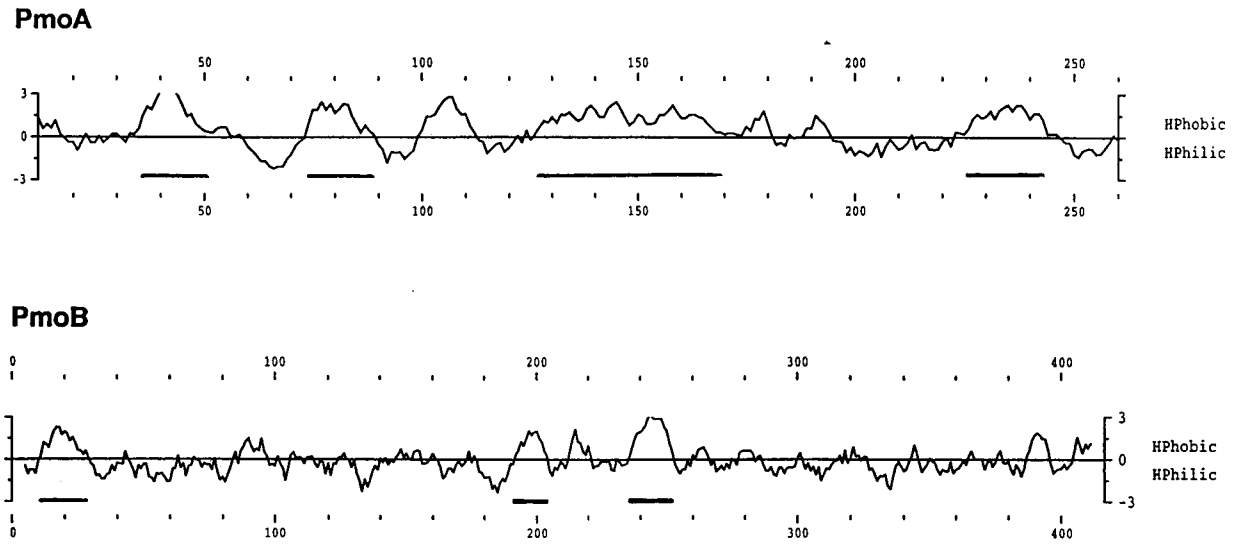

FIG. 7. Predicted hydropathy plots of the *pmoA* and *pmoB* gene products by the Kyte-Doolittle algorithm over a span of 7 residues. HPhobic, hydrophobic; HPhilic, hydrophilic. Scales above and below the plots indicate the numbers of residues. Bars denote predicted membrane-spanning sequences.

hybridization, suggesting that these sites are conserved in both gene copies. However, only one of the two possible *Pst*I fragments was cloned. For the *Sal*I sites, the restriction map shown in Fig. 3 predicts the hybridization data obtained, that the AC10 probe should identify only the 0.9-kb fragment, while the *Pst*I probe should identify in addition the 0.4- and 0.5-kb fragments. The weak hybridization of the 0.5-kb fragment is in accordance with the small (0.1-kb) overlap between this fragment and the *Pst*I probe. Therefore, all of the *Sal*I fragments identified are apparently present in the second gene copy also.

The same blots used for hybridization to the *pmoB* probe were washed and rehybridized to the *amoA* probe, but the hybridization to the *amoA* probe was weak and it was difficult to obtain accurate sizes for the hybridizing fragments. However, in the case of *Bam*HI, *Hin*dIII, *Eco*RI, and *Bst*YI, the data suggested that the same two fragments that hybridized to both *pmoB* probes also hybridized to *amoA*.

Hybridization of *Methylobacter albus* BG8 DNA with *amoA* and *pmoB* probes. Further hybridization experiments were carried out with *Methylobacter albus* BG8 DNA to obtain more information on the *pmo* genes in this strain. Seven different restriction digests were tested with the same probes used against the *Methylococcus capsulatus* Bath DNA. The 0.9-kb *Pst*I fragment hybridized to multiple bands in all cases, and a pattern of dark and light bands was observed (Fig. 5B; Table 1). The AC10 probe also showed a pattern of one light and one dark band for each digest (Fig. 2).

The same blots were washed and rehybridized with the *amoA* probe, and stronger hybridization was observed to the *Methylobacter albus* BG8 DNA with this probe than to the *Methylococcus capsulatus* Bath DNA (Fig. 5B). However, for each digest, only a single band was identified. In each case, this band corresponded to one of the bands that hybridized weakly to the *pmoB* probe (Table 1; Fig. 5B).

These data suggest that in *Methylobacter albus* BG8, there are two copies of *pmoB* and at least one copy of *pmoA*. This copy of *pmoA* is apparently linked to one of the copies of *pmoB*, as in *Methylococcus capsulatus* Bath. However, in this case, the copy of *pmoA* that was identified by the *amoA* probe was on the same DNA fragment as the copy of *pmoB* that is less similar to *pmoB* from *Methylococcus capsulatus* Bath.

DISCUSSION

The pMMO that oxidizes methane to methanol in methanotrophs is not well understood biochemically. Although some information is available concerning substrate range and inhibitors (8, 9, 23, 30), the pMMO is unstable and has never been purified reproducibly to homogeneity (39). Therefore, the structure of the enzyme is uncertain, and details concerning assembly and maturation processes are completely unknown. In this report, we describe the first steps towards a genetic approach to studying the pMMO. We have cloned and sequenced genes from *Methylococcus capsulatus* Bath that encode polypeptides with high similarity to the subunits of the AMO. The available data suggest that these genes are subunits of the pMMO, but further work will be necessary to confirm this hypothesis.

Our data suggest that *pmoA* and *pmoB* are present in duplicate gene copies in a variety of methanotrophs. In *N. europaea*, *amoA* and *amoB* apparently also exist in dual gene copies (26). In the case of *Methylobacter albus* BG8, light and dark hybridizing bands were observed when a *pmoB* probe was used, suggesting that one of the copies of *pmoB* in *Methylobacter albus* BG8 is more similar to the *pmoB* of *Methylococcus capsulatus* Bath than the other. The DNA fragment containing this less-similar *pmoB* copy of *Methylobacter albus* BG8 also appears to contain a copy of *pmoA* that has more similarity to *amoA* than the *pmoA* of *Methylococcus capsulatus* Bath has. A direct comparison must await the cloning and sequencing of both sets of genes from both methanotrophs. However, these data are intriguing and suggest that the two sets of *pmo* genes have diverged within the methanotrophs. Both *Methylobacter albus* BG8 and *Methylococcus capsulatus* Bath are phylogenetically related, being classed in the gamma subdivision of the proteobacteria, but they are in separate genera (5).

Whether both sets of genes are expressed in methanotrophs is not known. It has recently been shown that the pMMO exists in two kinetically and spectroscopically distinct forms, depending upon the copper concentration in the growth medium (23). At a high copper concentration, a low K_m form is expressed that has a specific electron paramagnetic resonance signature, while at a low copper concentration, a high K_m form is present

that lacks this electron paramagnetic resonance signature (23). It was originally suggested that these two forms of the pMMO might be generated posttranslationally, but the presence of two gene copies of *pmoA* and *pmoB* suggests the possibility that these two forms of the pMMO might be separate gene products.

The finding that the pMMO may be closely related to the AMO is not surprising, in light of the many common characteristics known for these enzymes. However, the finding that structural genes for both of these enzymes are present in two copies is more unexpected. It is relatively rare to find duplicate gene copies in prokaryotes, but several examples are known (26), and in some cases, the presence of two genes is the norm. For instance, the genes involved in carbon dioxide fixation (*cfx*) in facultative autotrophic bacteria are usually found in two sets (4). In those cases in which it has been studied, the duplicate sets of *cfx* genes are closely related in DNA sequence (up to 99% identical) and are apparently functionally equivalent (4). Our preliminary data for $pmoB_1$ and $pmoB_2$ of *Methylococcus capsulatus* Bath suggest that these genes are also closely related in DNA sequence. To determine the significance of these two gene copies of pMMO in the methanotrophs, it will be necessary to clone and sequence both sets of genes from a methanotroph and to generate and characterize mutants in each gene set. However, the results presented here raise the intriguing possibility that two different pMMOs might be present in methanotrophs and might be expressed differentially depending upon cellular needs.

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